USEFULNESS OF CORD BLOOD ANALYSIS IN PREDICTING PATHOLOGICAL HYPERBILIRUBINEMIA IN BABIES AT RISK OF DEVELOPING ABO INCOMPATIBILITY

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CERTIFICATE

Certified that this dissertation entitled **"USEFULNESS OF CORD BLOOD ANALYSIS IN PREDICTING PATHOLOGICAL HYPERBILIRUBINEMIA IN BABIES AT RISK OF DEVELOPING ABO INCOMPATIBILITY"** is a bonafide work done by **Dr. AJISH. T.P, M.D.,** Postgraduate student of Paediatrics Medicine, Institute of Child Health and Hospital for Children, Egmore, Chennai –8 attached to Madras Medical College, during the academic year 2005-2008.

DR. T. L. RATNAKUMARI, M.D., D.C.H.
Additional Professor Of Paediatrics – Neonatology,
H.O.D, Department of Neonatology,
Institute Of Obstetrics and Gynaecology,
Madras Medical College.

DR. S. BHAGAVATHY, M.D., D.C.H,
Additional Professor Of Paediatrics,
Institute Of Child Health and Hospital for Children
Madras Medical College

Prof. Dr. SARADHA SURESH, M.D, Ph.D., F.R.C.P. (Glas),
Director and Superintendent (I/C),
Institute of Child Health and Hospital for Children.
Madras Medical College,Chennai.

Prof. Dr. T. P. KALANITI, M.D.,
Dean,
Madras Medical College,
Chennai.

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CONTENTS

INTRODUCTION

Jaundice is the commonest abnormal finding in the first week of life. The clinical jaundice will manifest in neonates at a serum bilirubin level above 5.0 to 7.0 mg/dL (86 – 119 micromoles/L) [1]. Chemical hyperbilirubinemia, which is defined as serum total bilirubin level of 2.0 mg/dL (34micromoles/L) or more, is virtually universal in newborns during first week of life [1].

Between 25 – 50 % of all term newborns and a higher percentage of premature infants develop clinical jaundice. Also 6.1 % of well term newborns have a maximal serum bilirubin level > 12.9mg/dL. A serum bilirubin level of > 15mg/dL is found in 3 % of normal term babies [2]. As the intensity of jaundice increases, there is cephalocaudal progression of yellow discolouration of skin.

Hyperbilirubinemia can cause bilirubin encephalopathy and severe sequelae. So it is imperative that pathological hyperbilirubinemia is picked up early and vigorous treatment is started. When the newborn stays at the hospital for a 72-hour post-delivery period, it is possible to observe the peaking of the physiological jaundice, thus allowing medical intervention, if necessary. However, in cases of early discharge from the hospital, the newborns may be subjected to re-admission for phototherapy treatment because of high levels of unconjugated bilirubin [3]. Such re-admissions, besides involving extra expenses for both the family and the institution and also exposing a probably healthy newborn to the hospital environment, brings emotional problems and risks to breast-feeding, and is one of the causes of early weaning [4].

In the Institute of Obstetrics and Gynaecology there are 1800 deliveries conducted every month and most babies were discharged in 48 to 72 hours unless they are delivered by caesarean section or there are some maternal or newborn complications. 5-10% babies delivered are admitted in the newborn unit for neonatal hyperbilirubinemia, out of which the major number of cases are due to ABO incompatibility. So it is worth screening these babies who are at risk of developing ABO incompatibility. With this idea in mind this study is embarked upon.

BILIRUBIN METABOLISM

Bilirubin is a yellow breakdown product of normal heme catabolism. Bilirubin formed in utero by the fetus is cleared by the placenta. 75% of bilirubin is derived from the breakdown of hemoglobin and 25% from non hemoglobin sources like myoglobin and cytochrome.

Bilirubin is carried bound to albumin to the liver. In liver bilirubin is transferred across the cell membrane into the hepatocyte where it is bound principally to ligandin [5, 6], conjugated with two glucuronide molecules and excreted through the bile into the intestine[7]. In the presence of normal gut flora, the conjugated bilirubin is metabolized further to stercobilins and excreted in the stool. In the absence of gut flora and with slow intestinal motility, the conjugated bilirubin remains in the intestinal lumen, where a mucosal enzyme (beta-glucuronidase) can cleave off the glucuronide molecules, leaving unconjugated bilirubin to be reabsorbed (the enterohepatic circulation of bilirubin).

BILIRUBIN ALBUMIN BINDING

Unconjugated bilirubin is bound tightly to albumin at a primary (high-affinity) binding site as well as a secondary (low-affinity) site. Because binding affinity at the primary binding site is 10^7 to 10^8 L/mol, the concentration of free or unbound bilirubin in plasma is very low, even in the presence of significant hyperbilirubinemia [8]. Under normal circumstances 99.9% of the unconjugated bilirubin is bound to albumin. Free bilirubin is therefore present only in nanomole quantities. At lower pH bilirubin combines with H^+ and form bilirubin-H_2 which is water insoluble and forms complexes with phospholipids in cell membranes leading to the neuronal damage of kernicterus.

FACTORS AFFECTING BILIRUBIN BINDING TO ALBUMIN

Acid pH decreases the binding of bilirubin with albumin. Drugs like sulfonamides, moxalactam, fusidic acid, radiographic contrast media, aspirin and rapid infusion of ampicillin displaces bilirubin from albumin binding sites [1]. Long chain free fatty acids decrease the binding of bilirubin to albumin.

MEASUREMENT OF BILIRUBIN LEVELS

Although bilirubin estimation is one of the most commonly performed laboratory measurements in the newborn, its measurement remains remarkably inaccurate [9, 10]. Various methods can be used for the estimation of bilirubin.

Biochemical: Laboratory estimation of total and conjugated bilirubin based on Vanden Bergh reaction remains the gold standard for bilirubin estimation. Total and direct bilirubin levels can be measured from the blood, but indirect bilirubin is calculated from the total and direct bilirubin. Bilirubin is broken down by light when kept outside. But under the usual clinical conditions, there is no measurable effect of ambient light on serum bilirubin levels for at least 8 hours [11].

Bilimeter is based on spectro-photometry and estimates total serum bilirubin. It is useful in neonates, as bilirubin is predominantly unconjugated.

Transcutaneous bilirubinometer is non invasive and based on the principle of multi-wavelength spectral reflectance from the bilirubin staining in the skin. It could be used not only as a screening device but also as a reliable substitute for total serum bilirubin estimation [12].

MECHANISM OF HYPERBILIRUBINEMIA IN NEWBORN

Several physiological handicaps lead to increased frequency and severity of jaundice among newborn babies. Physiological polycythemia and shorter lifespan of fetal red blood cells (90 days vs. 120 days in adults) results in release of 0.15g/kg of hemoglobin every day as 1ml/kg of blood hemolyses every day. One gram of hemoglobin yields about 34 mg of bilirubin. Additional 1mg/kg of bilirubin is formed from non hemoglobin sources viz. myoglobin, cytochromes and catalases thus resulting in net daily load of about 20mg of bilirubin to the liver in a healthy term infant. Bilirubin production decreases with increasing postnatal age but is still about twice the adult rate by age 2 weeks [13].

Circulating bilirubin is bound to plasma albumin. It is believed that the neurotoxicity associated with hyperbilirubinemia is primarily a result of unbound or 'free' bilirubin. So the amount of albumin available for binding is important. The full term newborn infant has a significantly low level of plasma albumin compared to that for an adult and correspondingly less bilirubin binding sites. Adult levels are reached only by 5 months. Various drugs compete with bilirubin for binding site in albumin. Acid pH and free fatty acid decrease binding of bilirubin to albumin.

Hepatic uptake, conjugation and excretion of bilirubin are limited due to transient deficiency of Y and Z-acceptor proteins and UDP glucuronyl transferase enzyme in newborn babies especially those born prematurely. In the first 10 days after birth, UDP glucuronyl transferase enzyme activity in full-term and premature neonates is usually less than 1% of the adult values [14, 15]. Thereafter, its activity increases at an exponential rate, reaching adult values by 6 to 14 weeks of age [15].

Infants have fewer bacteria in their small and large bowel and greater activity of the deconjugating enzyme beta glucuronidase [16]. As a result conjugated bilirubin which is not reabsorbed is not converted to urobilinogen but is hydrolyzed to unconjugated bilirubin, which is reabsorbed, thus increasing the bilirubin load on an already stressed liver. Enterohepatic circulation of bilirubin is a significant contributor to physiologic jaundice [17]. In the first few days after birth, caloric intake is low, which contributes to an increase in the enterohepatic circulation [18, 19]. Thus increased production, reduced hepatic clearance and enhanced entero-hepatic circulation of bilirubin contributes to the increased prevalence of jaundice among newborn babies.

Multiple studies over the last 25 years have found a strong association between breast-feeding and an increased incidence of neonatal hyperbilirubinemia [20]. Although occasional studies have not proved this,[21,22] a pooled analysis of 12 studies of more than 8,000 newborns showed that breast-fed infants were three times more likely to develop total serum bilirubin levels of 12 mg/dL (205 µmol/L) or higher and six times more likely to develop levels of 15 mg/dL (257 µmol/L) or higher than formula-fed infants [23] . Ninety percent or more of infants readmitted to hospital in the first 2 weeks of life because of severe hyperbilirubinemia are fully or partially breast-fed [24, 25, 26]. A similar results were obtained from a study conducted in North California also [27].

The causes of hyperbilirubinemia in newborn period are shown in the table below.

HEMOLYTIC DISORDERS Rh and ABO incompatibility Hereditary spherocytosis G 6 PD deficiency Alpha thalassemia Acquired hemolysis due to drugs **EXTRAVASCULAR BLOOD** Petechiae, hematomas Pulmonary/cerebral/occult hemorrhage. **POLYCYTHEMIA** Feto maternal or feto-fetal transfusion Delayed clamping of umbilical cord **METABOLIC CAUSES** Galactosemia Criggler – Najar syndrome Gilbert's disease Hypothyroidism Tyrosinosis IDM Prematurity Breast milk jaundice	**MIXED OBSTRUCTIVE DISORDERS** Sepsis Intra uterine infections RDS Asphyxia IDM **INCREASED ENTEROHEPATIC CIRCULATION** Intestinal atresia Hirschprungs disease Meconium ileus Fasting hypoperistalsis Drug induced paralytic ileus Swallowed maternal blood

HEMOLYTIC JAUNDICE

Hemolytic disease in newborn period as a consequence of alloimmunisation of the mother is caused by the passage of fetal erythrocytes into the maternal circulation, in which they stimulate the production of antibodies. Antibodies of the IgG class return to the fetal circulation, attach to antigenic sites on the surface of the erythrocyte, and cause its rapid removal by the fetal reticuloendothelial system. The incidence and clinical manifestations of alloimmunisation depend on the type of blood group incompatibility between the mother and fetus [28, 29, 30]. The common causes of hemolytic jaundice in newborn include Rh hemolytic disease, ABO incompatibility, G-6-PD deficiency and minor blood group incompatibility.

The catabolism of erythrocytes results in the equimolar production of bilirubin and carboxyhemoglobin [31]. A rapid increase in the degree of hyperbilirubinemia is a cardinal sign of erythrocyte destruction. The measurement of blood carboxyhemoglobin or the rate of carbon monoxide excretion [32] correlates with the degree of hemolysis [33, 34].

RH HEMOLYTIC DISEASE

The incidence of Rh incompatibility in a population depends, in large part, on the prevalence of the Rh-negative antigens. The prevalence of the Rh-negative genotype ranges from approximately zero in Japanese, Chinese, and North American Indian populations to 5.5% among African-Americans and 15% among American Caucasians [35, 36]. Among Caucasian women, it has been estimated that in approximately 9% of all pregnancies, an Rh-negative woman carries an Rh-positive fetus. In 6% of pregnancies at risk, alloimmunisation of the mother occurs if there is no immunoprophylaxis [1].

The severity of Rh hemolytic disease varies greatly from infant to infant. It is estimated that, without antenatal diagnosis and treatment, the perinatal mortality in this disease would be approximately 17.5%, with stillbirths accounting for about 14% of deaths [30]. The degree of hemolytic disease tends to be more severe in subsequent pregnancies than in the initial one in which sensitisation had occurred.

Rh immunisation tends to occur more frequently in pregnancies that have been complicated by toxemia, cesarean section, or manual removal of the placenta, because transplacental hemorrhages occur with greater frequency and in greater volume under these circumstances. It is estimated that 1% of Rh-negative women develop antibodies as a consequence of these transplacental hemorrhages before the delivery of their first child. An additional 7.5% manifest evidence of sensitisation within 6 months of the delivery of their first child, and another 7.5% show no evidence of immunisation 6 months after delivery but develop antibodies during their next pregnancy if their fetus is Rh-positive, presumably as a consequence of a sensitisation during the first pregnancy.

ABO HEMOLYTIC DISEASE

Feto maternal ABO incompatibilities occur in about 20 – 25 % of pregnancies but severe hemolytic disease develop in only one in ten of such offspring. In ABO hemolytic disease of the newborn (also known as ABO HDN) maternal **IgG antibodies** with specificity for the **ABO blood group system** pass through the **placenta** to the **fetal** circulation where they can cause **hemolysis** of fetal **red blood cells** which can lead to fetal **anemia** and **hemolytic disease**.

Anti-A and anti-B antibodies are found in the IgA, IgM, and IgG fractions of plasma. Only the IgG antibodies cross the placenta and are responsible for the production of disease. These naturally occurring antibodies result from continuous immune stimulation by A and B substances that exist in foods and gram-negative bacteria. Anti-A and anti-B titers are low or absent in most pregnancies and it is not understood why some women develop high anti-A or anti-B titers. They may be the result of repeated, asymptomatic bacterial infections.

Fewer A or B antigenic sites are present on the erythrocytes of the newborn, which is responsible for the weakly reactive Coombs test in infants with ABO hemolytic disease. Significant jaundice in Direct Antiglobulin Test -negative neonates with **ABO** incompatibility (mother blood group O, infant A or B) is still often attributed to isoimmunisation. The sparse distribution of A and B sites on the erythrocytes of the newborn also explains why the erythrocyte life span in ABO hemolytic disease is only slightly shortened.

Before birth, the chief danger of excess erythrocyte destruction is profound anemia. After birth, the infant is primarily at risk from the toxic products of erythrocyte breakdown, such as bilirubin. In utero, the infant responds to the increased breakdown of cells by increasing the rate of erythrocyte production. This accelerated demand for erythrocytes results in active erythropoiesis in non-marrow sites such as the liver, spleen, and lung. A major portion of the hepatosplenomegaly observed in infants with hemolytic disease is a result of this extramedullary erythropoiesis.

BILIRUBIN TOXICITY

The effects of bilirubin toxicity are often devastating and irreversible. Acute bilirubin encephalopathy is classically seen in term infants dying of Rh hemolytic disease with high (>20 mg/dL) bilirubin levels who had kernicterus on autopsy. The AAP recommends [37] that the term acute bilirubin encephalopathy is used to describe the acute manifestations of bilirubin toxicity seen in the first weeks after birth and that the term kernicterus be reserved for the chronic and permanent clinical sequelae of bilirubin toxicity. Johnson and associates suggested the use of the term bilirubin-induced neurologic dysfunction (BIND) to describe the changes associated with acute bilirubin encephalopathy; they also proposed a scoring system to quantify the severity of the clinical manifestations[38].

The clinical presentation of bilirubin encephalopathy can be divided into three phases.

1. In the early phase, the infant becomes lethargic and hypotonic, and sucks poorly.

2. The intermediate phase is characterized by moderate stupor, irritability, and hypertonia. The infant may develop a fever and a high-pitched cry [39], which might alternate with drowsiness and hypertonia.

3. The advanced phase is characterized by pronounced retrocollis-opisthotonus, shrill cry, refusal to feeds, apnea, fever, deep stupor to coma, and sometimes seizures and death [39, 40, 41].

Subsequently, usually after 1 week, hypertonia subsides and is replaced by hypotonia. In the first year, infants typically feed poorly, develop a high-pitched cry, and are hypotonic, but have increased deep tendon reflexes, a persistent tonic neck reflex, and motor delay [42]. There is a delay in acquisition of motor skills, although most children walk alone by 5 years of age. The other typical features of chronic bilirubin encephalopathy usually are not apparent before 1 year of age and often not for several years [39]. These children generally are hypotonic at rest for the first 6 or 7 years. By the time they reach their teens, hypertonia will replaced hypotonia [43].

Chronic bilirubin encephalopathy constitutes a tetrad consisting of extra pyramidal disturbances, auditory abnormalities, gaze palsies, and dental dysplasia [43].

The term "Kernicterus" refers to the neurologic consequences of the deposition of unconjugated bilirubin in brain tissue. Full-term infants who die of kernicterus demonstrate bilirubin staining in a characteristic distribution. The regions most commonly affected are the basal ganglia, particularly the sub thalamic nucleus and the globus pallidus, the hippocampus, the geniculate bodies, various brainstem nuclei, including the inferior colliculus, oculomotor, vestibular, cochlear, and inferior olivary nuclei, and the cerebellum, especially the dentate nucleus and vermis [44, 45]. Subsequent damage and scarring of the basal ganglia and brain-stem nuclei may occur [46].

The exact bilirubin concentration associated with kernicterus in a healthy term infant is unpredictable. Toxicity levels may vary among ethnic groups, with maturation of an infant, and with the presence of hemolytic disease. Although the risk of bilirubin toxicity is probably negligible in a healthy term newborn without hemolysis, the physician should become concerned if the bilirubin level is above 25 mg per dL (428 μmol per L). In the term newborn with hemolysis, a bilirubin level above 20 mg per dL (342 μmol per L) is a concern.

SCREENING OF BABIES FOR ALLO IMMUNISATION

NEED FOR SCREENING

In the era of early discharge of newborns from the hospital, newborns with ABO incompatibility are especially at greater risk for developing a significant hyperbilirubinemia after discharge and a considerable percentage of these could present with isoimmunisation (ABO). Early prediction of a likelihood of pathological hyperbilirubinemia could decrease the need for exchange transfusion, which also can cause some complications. The use of some screening tests have significantly decreased the incidence of exchange transfusion in developed countries.

ANTENATAL SCREENING FOR ALLO IMMUNISATION

All pregnant women should be tested for ABO and Rh (D) typing and undergo a serum screen for unusual isoimmune antibodies [37]. In infants of group O or Rh-positive mothers, the AAP recommends that routine testing for blood type and Coombs test is optional provided there is appropriate surveillance and risk assessment before discharge and follow up so that significantly jaundiced infants are not missed.

ANTIBODY TITERS

Serial antibody titers are the mainstay of maternal assessment. After it is determined that there is maternal sensitisation and the possibility of fetal antigen positivity, additional antibody titer determinations should be performed as the pregnancy progresses. A second maternal antibody titer is performed at 18 to 20 weeks of gestation and then repeated monthly for the rest of the pregnancy. Rarely does significant hemolytic disease in the fetus occur before 20 weeks of gestation. Those patients who have a positive antibody screen for the Dd antigen, or for one of the atypical antigens that are capable of causing significant hemolytic disease of the newborn, need close follow up.

Severe hemolytic disease of the newborn rarely occurs when the Coombs titer is less than 1:16. Therefore, patients whose antibody titers are below this level can be managed expectantly and delivered at term with little change in routine obstetric care.

Patients whose antibody titer in the current or any previous pregnancy has exceeded 1:16 and who are carrying a potentially antigen-positive fetus must be considered at risk for severe hemolytic disease of the newborn.

FETAL ASSESSMENT BY AMNIOCENTESIS

Fetal antigen status can be inferred from information gained from antigen testing of the father. When necessary, fetal antigen status can be determined directly by cord-blood sampling or from fetal cells obtained by amniocentesis. Fetal anemia can be diagnosed from fetal blood obtained from cord-blood sampling. In general, in a first affected pregnancy, hydrops fetalis is unlikely before 26 to 28 weeks, and therefore invasive procedures to assess the degree of fetal anemia can usually be delayed until that time. In patients who have previously had a severely affected fetus, fetal testing is usually started 1 to 4 weeks before the time the fetus was noted to be severely affected in the previous pregnancy.

Traditionally, amniocentesis with analysis of amniotic fluid for breakdown products of hemoglobin has been used to identify fetuses likely to be severely anemic. In addition, fetal blood sampling can be used to assess the fetal antigen status and degree of anemia in affected pregnancies.

ULTRASOUND

Ultrasound evaluation of the fetus is a noninvasive method of evaluating the fetus at risk for significant anemia and hydrops. Hydrops is relatively easy to identify by ultrasound. Increased amniotic fluid volume, placental thickening, and hepatosplenomegaly are ultrasound signs of early hydrops.

DOPPLER STUDIES

Recently, a number of studies have demonstrated that the degree of fetal anemia can be accurately predicted by Doppler studies of blood flow in the fetal middle cerebral artery (MCA). It appears likely that MCA Doppler will replace the other more traditional methods of fetal assessment because it is noninvasive, widely available, easy to repeat regularly, and well-accepted by the mothers[47, 48, 49] Animal and human studies have demonstrated that blood flow in the brain is increased in fetuses with anemia because of an increase in cardiac output and decrease in blood viscosity[50, 51]

The MCA is identified using color flow Doppler ultrasound, and duplex Doppler is then used to estimate the peak systolic velocity. Normal values for peak systolic velocity in the MCA have been established for fetuses between 18 and 36 weeks of gestation. Fetuses with peak systolic velocity <1.5 multiples of the median are at low risk for significant anemia [48].Fetuses with middle cerebral artery Doppler estimated velocity >1.5 multiples of the median are considered at risk for severe anemia and further investigated by either fetal blood sampling or amniotic fluid bilirubin studies. MCA Doppler studies pose no risk to the pregnancy, and serial testing (every 2 to 4 weeks) is typically begun at 20 to 24 weeks.

LILEY GRAPH

In 1961 Liley [52] described a method of amniocentesis and amniotic fluid spectrophotometric analysis for predicting hemolytic disease.

The optical density reading at 450 nm (OD_{450}) is directly related to the severity of hemolytic disease. By plotting OD_{450} versus gestational age, Liley was able to evaluate the degree of hemolytic disease in 101 Rh-immunised pregnancies. This initial work performed on gestations after 28 weeks allowed prediction of the clinical course based on the arbitrary division of the patients into three zones. Severe hemolytic disease, fetal hydrops, and fetal death were temporally related to readings in the upper zone (zone 3). Mild or no hemolytic disease was correlated with zone 1. Empirical data and developing prognostic accuracies have evolved the graph to its current form. The method of measurement used, however, seems less important than the judgment and experience of the person reviewing the amniotic fluid results.

FETAL TO MATERNAL HEMORRHAGE ESTIMATION

The presence of fetal red cells in the maternal circulation may be identified by use of the acid elution principle first described by Kleihauer, Brown, and Betke, or any of several modifications. This test is based on the fact that fetal erythrocytes contain hemoglobin F, which is more resistant to acid elution than hemoglobin A. After exposure to acid, only fetal hemoglobin remains. Fetal red cells can then be identified by uptake of a special stain and quantified on a peripheral smear.

SCREENING OF NEWBORNS

Post natal screening of infants at risk is by cord blood analysis, serum bilirubin monitoring, non invasive bilirubin monitoring and end tidal carbon monoxide monitoring.

CORD BLOOD ANALYSIS

Decreased hemoglobin concentration, increased reticulocyte count, and increased numbers of nucleated erythrocytes in the peripheral blood reflect the presence of the hemolytic process. Hemoglobin determination performed on venous samples most accurately reflects the severity of the hemolytic process. Values less than 13 g/dL in the cord blood should be regarded as abnormal.

The reticulocyte count is usually greater than 6% and may reach 30% to 40%. In the peripheral blood, nucleated erythrocytes may be observed in addition to some degree of polychromasia and anisocytosis. A baby born to an Rh-negative mother (and Rh-positive father) should have Rh typing and a Direct Coomb's test (DCT) on cord blood. Newborns suspected to have Rh isoimmunisation should have a blood group and Rh typing, Direct Coombs Test, PCV and serum bilirubin on cord blood to facilitate early treatment. It has been suggested that cord hemoglobin less than 11.0 g/dL or a cord bilirubin higher than 4.5 mg/dL is an indication for an immediate exchange transfusion [30]. Intensive phototherapy should be started at birth and continued till a level, which is 5 mg/dl less than that for exchange blood transfusion. Prophylactic phototherapy may be beneficial and is indicated when the cord bilirubin is at least 4 mg/dl [53].

As there is very little anti-A or anti-B antibody on the neonatal red blood cell in **ABO** hemolytic disease, the cord blood direct antiglobulin test in this condition is only weakly positive and may be negative unless a sensitive test is used. One currently accepted method in hospital l**abo**ratories for performing the direct antiglobulin test is a gel test that is more sensitive than formerly used techniques for detecting immunoglobulin G coating of newborn red cells.

At birth the ABO hemolytic disease can be suspected by examining the cord blood for elevation of serum bilirubin, presence of maternal IgG anti A or anti B antibodies in an antibody-dependant cell mediated cytotoxicity (ADCC) assay and detection of antigen density of A or B antigens on the red blood cells. Reticulocytosis, spherocytosis and increased osmotic fragility of RBCs may be present. The diagnosis of ABO hemolytic disease is supported by the finding of increased numbers of spherocytes; these are best detected by evaluating the three-dimensional shape of the erythrocyte. Increased erythrocyte production is also demonstrated by an increased reticulocyte count.

The levels of IgG anti-A or anti-B in the mothers of babies with ABO hemolytic disease is significantly higher than those in mothers whose infants do not have the disease. These tests are often not available and, unfortunately, are not high in specificity or sensitivity in the diagnosis of ABO hemolytic disease. The nucleated RBC count is elevated and falsely elevates the leukocyte count, reflecting a state of erythropoiesis. Spherocytosis (<40%) is more commonly observed in cases of ABO incompatibility. In severe hemolytic disease, schistocytes and burr cells may be observed, reflecting ongoing disseminated intravascular coagulation. A low reticulocyte count is observed in fetuses provided with intravascular transfusion in utero and with Kell alloimmunisation.

Neutropenia seems to be secondary to stimulation of erythropoiesis in favor of myelopoiesis

SERUM BILIRUBIN MONITORING

Vinod K. Bhutani, Ron Keren, MD, et al [54], Department of Pediatrics, University of Pennsylvania School of Medicine, Philadelphia, suggested the hour-specific total serum bilirubin before discharge as the most accurate method for assessing risk of severe hyperbilirubinemia. A serum bilirubin measurement and the use of the critical bilirubin levels of 4 mg/dL and 6 mg/dL at the sixth hour of life will predict nearly all newborns who will have significant hyperbilirubinemia and those who will develop severe hemolytic disease of the newborn. Non invasive monitoring of serum bilirubin using trans cutaneous bilirubinometers is useful in screening of neonates at risk.

END TIDAL CARBONMONOXIDE MONITORING

The degradation of hemoglobin results in the equimolar formation of bilirubin and carbon monoxide. After formation, the carbon monoxide binds to hemoglobin within circulating red blood cells forming carboxyhemoglobin. The carboxyhemoglobin dissociates in the lung releasing carbon monoxide into the exhaled breath. The concentration of carbon monoxide present in the alveoli of the lungs is directly related to the concentration of carboxyhemoglobin within the blood. The measurement of blood carboxyhemoglobin or the rate of carbon monoxide excretion [55] correlates with hemolysis [56,57,58].

MANAGEMENT OF PATHOLOGICAL HYPERBILIRUBINEMIA

Before treatment is initiated, the minimum evaluation should include the infant's age and postnatal course, a maternal and gestational history, physical examination of the infant, and determination of the total serum bilirubin level and the rate at which it is rising. Jaundice in a term newborn fewer than 24 hours old is always pathologic. It should be investigated thoroughly and treated appropriately. Conjugated hyperbilirubinemia is never physiologic, and it may indicate the presence of a potentially serious underlying disorder. If jaundice persists for more than two weeks in a formula-fed infant and more than three weeks in a breastfed infant, further evaluation is warranted.

Laboratory studies should include a fractionated bilirubin level, thyroid studies, evaluations for metabolic disorders or hemolytic disease, and an assessment for intestinal obstruction for a selected case.

Depending on the rate at which the bilirubin level rises, a newborn's risk of developing significant hyperbilirubinemia can be classified as low, intermediate, or high.

AAP gives guidelines for phototherapy and exchange transfusion in hospitalized infants of 35 or more weeks' gestation [37].

Hyperbilirubinemia can be treated in three ways:

a. Exchange transfusion removes bilirubin mechanically

b. Phototherapy converts bilirubin to products that can bypass the liver's conjugating system and be excreted in the bile or in the urine without further metabolism

c. Pharmacologic agents that interfere with heme degradation and bilirubin production, accelerate the normal metabolic pathways for bilirubin clearance, or inhibit the enterohepatic circulation of bilirubin.

Phototherapy is the most common treatment in use for hyperbilirubinemia; exchange transfusions generally are reserved for phototherapy failures [59]. The bilirubin level at which intervention is necessary is still a contentious issue. Treatment guidelines must rely on relatively uncertain estimates of risks and benefits and the recognition that using a single serum bilirubin level to predict long-term behavioral and developmental outcomes is not reliable and will lead to conflicting results [60].

PHOTOTHERAPY

Phototherapy was discovered rather serendipitously in England in the 1950s and now is arguably the most widespread therapy of any kind (excluding prophylactic treatments) used in newborns. The main demonstrated value of phototherapy is that it reduces the need for exchange transfusion [61, 62].

Phototherapy is effective because three reactions can occur when bilirubin is exposed to light, as follows:

1. Photo oxidation
2. Configurational isomerisation
3. Structural isomerisation

Bilirubin can be photo-oxidised to water-soluble, colourless products that can be excreted in the urine. This is a slow process and is probably only a minor contributor to the elimination of bilirubin during phototherapy.

Configurational isomerisation is a very rapid process that changes some of the predominant 4Z, 15Z bilirubin isomer to water-soluble isomers in which one or both of the intramolecular bonds are opened (E, Z; Z, E; or E, E). In human infants, the 4Z, 15E isomer predominates, and at equilibrium conditions, the isomer constitutes about 20% of circulating bilirubin after a few hours of phototherapy. This proportion is not influenced significantly by the intensity of light. Thus, although configurational isomerisation is extremely rapid and accounts for the bulk of the photochemical outcomes, it probably plays only a minor role in lowering the serum bilirubin concentration because although it is formed fastest, the clearance of the light-generated 4Z, 15E isomer is very slow (T½ ~15 hours)[61].

Structural isomerisation consists of intramolecular cyclisation, an irreversible process, resulting in the formation of lumirubin. This process is enhanced by increasing the intensity of light. During phototherapy, lumirubin may constitute 2-6% of the total serum bilirubin concentration, which is much lower than the concentration of the configurational isomers that form about 20% of the total bilirubin. The photo isomers of bilirubin are excreted in bile and, to some extent, in urine [63].

The efficacy of phototherapy depends on several important factors.

1. Spectrum of light emitted
2. Irradiance of light source
3. Design of phototherapy unit
4. Surface area of infant exposed to the light
5. Distance of infant from light source

Blue-green spectrum is most effective; at these wavelengths, light penetrates skin well and is absorbed maximally by bilirubin. Irradiance or energy output may be increased in a phototherapy unit by decreasing the distance to within 15-20 cm of the infant. The ideal configuration is four special blue bulbs (F20T12/BB) placed centrally, with two daylight fluorescent tubes on either side. The power output of the lights (irradiance) is directly related to the distance between the lights and the newborn. To expose the greatest surface area, the newborn should be naked except for eye shields. Double surface phototherapy may be more effective than single surface phototherapy [64].

The only contraindication to the use of phototherapy is conjugated hyperbilirubinemia, as occurs in patients with cholestasis and hepatic disease. In this setting, phototherapy may cause a dark grayish-brown discoloration of the skin (bronze baby syndrome) [65]. Potential problems that may occur with phototherapy include burns, retinal damage, thermoregulatory instability, loose stools, dehydration, skin rash, and tanning of the skin [66]. Because phototherapy is continuous, treatment also involves significant separation of the infant and parents [67].

With intensive phototherapy, the total serum bilirubin level should decline by 1 to 2 mg per dL (17 to 34 μmol per L) within four to six hours. The bilirubin level may decline more slowly in breastfed infants (rate of 2 to 3 mg per dL per day) than in formula-fed infants. Phototherapy usually can be discontinued when the total serum bilirubin level is below 15 mg/dL. The average rebound elevation of bilirubin level after phototherapy is below 1 mg per dL. Therefore, hospital discharge of most infants does not have to be delayed to monitor for rebound elevation.

Continuous phototherapy is better than intermittent phototherapy. Phototherapy should be interrupted in a newborn only during breast-feeding and nappy changes. In case of hemolytic or rapidly rising bilirubin or when a conventional unit is not effective, use of intensive phototherapy is warranted. This can be achieved by placing the infant on bili-blanket and using additional overhead phototherapy units with special blue lights and lowering the phototherapy units to within a distance of 15-20 cm.

When used properly, phototherapy is a very effective and safe method of lowering the serum bilirubin concentration. Its use has drastically decreased the need for exchange transfusion and it has probably contributed to the virtual disappearance of kernicterus in the low-birth-weight infants.

EXCHANGE TRANSFUSION

Exchange transfusion is the most rapid method for lowering serum bilirubin concentrations. This treatment is rarely needed when intensive phototherapy is effective [68]. The procedure removes partially hemolysed and antibody-coated erythrocytes and replaces them with uncoated donor red blood cells that lack the sensitising antigen. During exchange transfusion, bilirubin from the extra vascular space is drawn into the plasma, and partial equilibration between extra vascular and plasma bilirubin occurs almost instantaneously [69]. Thus, by the end of a double-volume exchange, in which only about 15% of the circulating erythrocytes remain, the serum bilirubin is still 45% to 60% of the pre exchange level [69, 70]. Immediately after the exchange, further equilibration takes place, which is completed within 30 minutes and produces the early rebound of plasma bilirubin to 60% to 80% of the pre exchange level [69].

In the presence of hemolytic disease, severe anemia, or a rapid rise in the total serum bilirubin level (greater than 1 mg per dL per hour in less than six hours), exchange transfusion is the recommended treatment. Exchange transfusion should be considered in a newborn with nonhemolytic jaundice if intensive phototherapy fails to lower the bilirubin level.

Complications of exchange transfusion can include air embolism, vasospasm, infarction, infection, and even death [60]. Because of the potential seriousness of these complications, intensive phototherapy efforts should be exhausted before exchange transfusion is initiated.

PHARMACOLOGICAL TREATMENT

Phenobarbitone: Phenobarbital is a potent inducer of microsomal enzymes that increases bilirubin conjugation and excretion and increases bile flow. When given in sufficient doses to the mother, the infant, or both, Phenobarbital is effective in lowering serum bilirubin levels in the first week of life [71, 72]. However, concerns about long-term toxicity when given to pregnant women militate against its use for this purpose [73]. When used prophylactically in a dose of 5 mg/kg for 3-5 days after birth, it has shown to be effective in babies with hemolytic disease, extravasated blood and in preterms without any significant side effects.

Intravenous Immunoglobulins (IVIG): Controlled trials have confirmed that the administration of IVIG to infants with Rh hemolytic disease will significantly reduce the need for exchange transfusion [74]. It also is likely that IVIG will help to mitigate the course of severe ABO hemolytic disease [75] and other isoimmune causes of hemolysis. The doses used have ranged from 500 mg/kg given over 2 hours soon after birth to 800 mg/kg given daily for 3 days. In Rh hemolytic disease, anti-D-coated erythrocytes are removed from the circulation through antibody-dependent lysis by cells of the reticuloendothelial system. The mechanism of action of IVIG is unknown, but it is possible that it might alter the course of Rh hemolytic disease by blocking Fc receptors, thereby inhibiting hemolysis. The risks of IVIG therapy are certainly lower than those of exchange transfusion.

Metalloporphyrins: Certain synthetic metalloporphyrins are powerful competitive inhibitors of heme oxygenase and suppress the formation of bilirubin. The inhibition of heme degradation to bilirubin does not result in the accumulation of heme; the heme is excreted unchanged in the bile in quantities that compensate for the decreased excretion of bilirubin [72]. These compounds are still experimental but showing promise in various hemolytic and non-hemolytic settings without significant side effects.

FOLLOW-UP

Babies with serum bilirubin >20 mg/dL and those who require exchange transfusion should kept under follow-up in the high- risk clinic for neuro-developmental outcome. Hearing assessment (BERA) should be done at 3 months of corrected age.

REVIEW OF LITERATURE

ABO incompatibility is a major cause of neonatal hyperbilirubinemia and neonatal hyperbilirubinemia if untreated is associated with significant morbidity and mortality. So it is important to screen these at risk infants. Various studies have been undertaken in an effort to identify an effective predictor of future neonatal hyperbilirubinemia in babies at risk of ABO incompatibility. These studies are discussed in following paragraphs.

The exact incidence of ABO hemolytic disease is not known. The incidence in various studies range from 3.7% (Han P, Kiruba R, etal [73]) to 32.95 % (Chen JY, Ling UP et al [74]). This marked difference may be due to racial variation, inter laboratory variation in bilirubin estimation or due to the criteria adopted for diagnosing ABO hemolytic disease.

Various parameters in the cord blood studied for prediction of pathological hyperbilirubinemia in ABO incompatibility include cord blood bilirubin, cord blood reticulocyte count, direct coomb's test, cord blood hemoglobin, end tidal carbon monoxide estimation etc.

J. Maxwell Johnstone [75], analysed cord blood samples of 1459 babies born in West Middlesex Hospital and found out that the mean serum bilirubin values in cord blood samples were 1.27 mg /dL. The mean cord blood bilirubin values of ABO heterospecific pregnancies

(1.36 mg/dL) was found to be significantly higher than the homospecific pregnancies (1.24 mg/dL).

Procianoy RS et al. [76] from Brazil assessed the usefulness of direct Coombs test in diagnosing ABO-hemolytic disease of the newborn in 132 term, AGA neonates. His study suggested that bilirubin concentration in the cord blood ≥ 4 mg/dL is useful for an early diagnosis of ABO-hemolytic disease. In a study conducted by **Whyte J, Graham H** [77] on seventy-one ABO incompatible infants concluded that cord serum bilirubin concentration had some predictive value, particularly when the level exceeded 85 micro mol/l, but it was a less reliable indicator and had greater value if used in association with the direct anti globulin test..

Chen JY, Ling UP et al [74] conducted a study in Taiwan in eighty-eight healthy full-term newborn infants born to blood group O mothers and concluded that maternal IgG anti-A or anti-B titers ≥ 512X, cord bilirubin levels ≥ 4 mg/dL or positive direct Coombs' test of the cord blood represent a "high risk" category, and should be placed in hospital where frequent re-evaluation and appropriate therapy are available.

Levine DH, Meyer HB [78] analysed 1391 cord blood specimens and found out that cord blood bilirubin was not diagnostic of hemolytic disease but was moderately predictive of peak bilirubin levels. Study conducted by **Han P, Kiruba R, etal** [73]**,** National University of Singapore on 1608 babies also suggested that cord bilirubin was of low predictive value for severe hemolytic disease due to ABO incompatibility. These data support the notion that it is not cost effective to screen for ABO incompatibility.

Vinod K. Bhutani, **Ron Keren, MD, et al** [54]**,** Department of Pediatrics, University of Pennsylvania School of Medicine, Philadelphia, suggested that the hour-specific total serum bilirubin before discharge has been shown to be the most accurate method for assessing risk of severe hyperbilirubinemia. Transcutaneous bilirubin and end-tidal carbon monoxide measurements and screening for specific genetic markers of neonatal hyperbilirubinemia have the potential to refine risk assessment strategies further.

Knupfer M, **Pulzer F** **et al** [79] investigated the predictive value of umbilical cord serum bilirubin for the postnatal course of bilirubinaemia in healthy term and near-term newborns and found out that umbilical cord serum bilirubin values correlated with the development of hyperbilirubinemia and phototherapy requirement. They concluded that a cut off value of 30 micromol/litre of cord bilirubin can be useful for predicting phototherapy requirement.

Various studies assessed the usefulness of direct coomb's test in babies with ABO incompatibility. The usefulness of direct coombs test in predicting significant hyperbilirubinemia is controversial. But most of the studies prove that even though coomb's test may be negative in ABO hemolytic disease coomb's test if positive may indicate a risk of severe hemolysis.

Study conducted by **Dufour DR**, **Monoghan WP** **etal** [80] in 254 babies with ABO hemolytic disease found out that sixty-five per cent of the infants who had positive direct antiglobulin tests experienced jaundice, compared with approximately 35% of control infants or infants who had ABO hemolytic disease of the newborn with negative direct antiglobulin test results. Infants who had ABO hemolytic disease of the newborn with positive direct antiglobulin test results also had greater severity of jaundice than control infants or infants who had ABO hemolytic disease of the newborn with negative direct antiglobulin test results. **Marcello Orzalesi M.D., Fulvia Gloria Math.D etal** [81] Department of Pediatrics, Yale University School of Medicine, Rome, Italy studied newborn infants incompatible with their mothers in the ABO system and found out that there is a potentially higher risk of severe hyperbilirubinemia particularly in the presence of a positive direct Coombs test.

Study conducted by **Whyte J**, **Graham H** [77] on seventy-one ABO incompatible infants concluded that modified Direct Antiglobulin Test (spin DAGT) was positive in all infants who required treatment for hemolytic jaundice and only DAGT positive children showed evidence of impending hemolytic anaemia. The elution test, which is frequently used as a diagnostic tool in ABO hemolytic disease, had no predictive value and they felt that its putative value is due to over-diagnosis of ABO hemolytic disease. **Chen JY, Ling UP et al** [74] conducted a study in Taiwan in eighty-eight healthy full-term newborn infants born to blood group O mothers and concluded that ABO incompatible newborn infants with positive direct Coombs' test of the cord blood represent a "high risk" category, and should be placed in hospital where frequent re-evaluation and appropriate therapy are available.

Procianoy RS et al. [76] from Brazil assessed the usefulness direct Coombs test in diagnosing ABO-hemolytic disease of the newborn in 132 term, AGA neonates. His study suggested that the direct Coombs test in combination with quantitative elution test and bilirubin concentration in the cord blood is useful for an early diagnosis of ABO-hemolytic disease.

Bel omos J et al, Barcelona [82].studied the ABO hemolytic disease of the newborn and found out that direct antiglobulin test was positive in 11.3% of them. The Elution was positive in all the newborns with direct antiglobulin test positive. They concluded that the direct antiglobulin test is very useful to detect the newborns liable to get serious jaundice. Levine DH, Meyer HB [78] analysed 1391 cord blood specimens for type, Rh, direct antiglobulin test, indirect Coombs, and total and indirect bilirubin. They found out that direct antiglobulin test was neither diagnostic nor predictive of severity. They concluded that the data does not support the use of any routine screening tests in the management of

ABO hemolytic disease.

Han P, **Kiruba R**, **etal** [73], National University of Singapore conducted a study on 1608 babies. The baby-maternal pairs were typed for ABO, Rh, and tested for direct Coombs' test, maternal titer, cord blood bilirubin and haptoglobin levels. They found out that the incidence of hemolytic disease due to ABO incompatibility was 3.7% of all group O mothers. Coombs' test, maternal antibody titre, cord bilirubin and haptoglobin levels were of low predictive value for severe hemolytic disease due to ABO incompatibility. The data further support the notion that it is not cost effective to screen for ABO incompatibility

Dinesh D [83], New Zealand Blood Service, estimated the positive predictive value of a positive direct antiglobulin test for hemolytic disease is 23%. The sensitivity was estimated to be 86%. Recommendations for testing are discussed but remain controversial in practice.

Marguerite Herschel, **MD et al** [84] studied babies with risk of ABO incompatibility and compared those who are direct antiglobulin test positive with the negative ones and found out that in direct antiglobulin test-negative newborns with significant jaundice or increased bilirubin production, even if ABO-incompatible, a cause other than isoimmunisation should be sought.

Molly Ann Katz, MD, William p. Kanto Jr. M.D et al [85] Medical

College of Georgia, Augusta studied 230 neonates with ABO hemolytic disease (HD) and concluded that there is no significant difference in clinical severity between AO-HD and BO-HD as measured by number of neonates with hyperbilirubinemia and/or those requiring exchange transfusion; hemoglobin concentration; reticulocyte count; bilirubin concentration; and incidence of anemia after discharge from the hospital.

S. Umit Sarici, MD [86] Turkey found out that a positive direct antiglobulin test, along with reticulocyte count and serum bilirubin is a good predictor for the development of significant hyperbilirubinemia and severe hemolytic disease of the newborn in ABO incompatibility.

Most of the studies done across the world concluded that cord blood analysis is useful for screening babies at risk of ABO incompatibility for pathological hyperbilirubinemia. Studies conducted by Levine DH, Meyer HB [78] and Han P, Kiruba R, etal [73] found out that routine screening of cord blood is not useful and cost effective. Direct coomb's test though has a low sensitivity, is useful in predicting pathological hyperbilirubinemia in most of studies[80, 85, 76, 82, 86]. Low cord hemoglobin levels and reticulocytosis was used a predictor of significant hemolysis and severity of ABO hemolytic disease rather than a screening test.

JUSTIFICATION OF STUDY

The earlier studies have been undertaken in an effort to identify early predictors of neonatal hyperbilirubinemia. In Tamilnadu nearly than 80% of the deliveries take place in hospitals. The trend in these hospitals is early discharge of mother and infant in 24-72hrs unless mother has undergone caesarean section or puerperal sterilization. Very few come for follow up and there is no proper home visit by field health worker to these mothers and infants.

The common reason for readmission of newborn in early neonatal period is jaundice. Another important fact to be considered is that the peak serum bilirubin is attained on the 3rd or 4th day of life in a term infants. This may lead to development of bilirubin encephalopathy at home. By the time these infants are brought to the neonatal care unit, they might have crossed the stage of salvation which leads to increased morbidity and mortality. From this it is evident that a prediction for the development of pathological jaundice is highly essential to decrease the morbidity and mortality due to bilirubin encephalopathy. A large majority of babies admitted with neonatal hyperbilirubinemia will have ABO incompatibility. It may not be feasible to screen all infants born for neonatal hyperbilirubinemia. But screening of babies who are at risk of developing hyperbilirubinemia due to ABO incompatibility is justifiable.

Most studies done in our country aims at prediction of ABO-hemolytic disease by cord bilirubin alone. Cord bilirubin alone is a poor predictor of ABO-hemolytic disease. A combination of cord bilirubin, direct coomb's test, reticulocyte count, cord hemoglobin may act as a very good predictor of ABO-hemolytic disease. A baby may be safely discharged at 48+/-hrs when all the above tests are negative thus decreasing the hospital stay if study proves it effective. Thus there is economic benefit and decreases risk of nosocomial infection in newborn and mother. If cord blood values prove to be useful in predicting ABO-hemolytic disease, it will be an early non invasive screening method. Early diagnosis of ABO-hemolytic disease is useful in peripheral hospitals where phototherapy units are not available.

Various studies conducted across the world have showed the critical cord serum bilirubin levels for development of pathological hyperbilirubinemia around 1.7 mg % to 5 mg% [74 – 88]. It was therefore proposed to find out the level of cord bilirubin in our setup beyond which our infants are at increased risk of developing pathological hyperbilirubinemia. Usefulness of other cord blood tests like reticulocyte count, cord blood hemoglobin and direct coomb's test are debatable.

OBJECTIVES OF THE STUDY

To study:

A. Whether cord blood analysis (cord bilirubin, reticulocyte count, hemoglobin and direct coomb's test) can serve as a useful predictor in a newborn at risk of ABO incompatibility for developing pathological hyperbilirubinemia.
B. Appropriate level of cord bilirubin, hemoglobin and reticulocyte count which can pick up maximum number of newborns who would go for pathological hyperbilirubinemia.

SUBJECTS AND METHODS

a) **STUDY DESIGN**: Descriptive study

b) **STUDY PERIOD**: 18 months (from January 2006 to June 2007)

c) STUDY POPULATION:

Babies born of caesarean section to O positive mothers during the period from January 2006 to June 2007 in the Institute of Obstetrics and Gynaecology were randomly selected for the study. Babies born of caesarean section were included in the study because these babies were usually kept in the hospital for a period of 10 days and they can be followed up during that period for the presence of jaundice.

d) OUTCOME MEASURES:

Serum bilirubin taken in the 4th day is taken as the outcome measure. Avery gives the criteria for pathological jaundice [1] as

- Jaundice within 24hours of life.
- Jaundice persisting >1 week.
- Total serum bilirubin more than the 95th percentile for the age in hours.
- Direct bilirubin >1.5 mg/dl.
- Total serum bilirubin increasing more than 5 mg/dl/day or 0.5 mg/dl/hour.

In our study pathological hyperbilirubinemia is defined as a serum total bilirubin level >15 mg/dL on 4th day of life or any total serum bilirubin more than the 95th percentile for the age in hours taken [37]. See figure below.

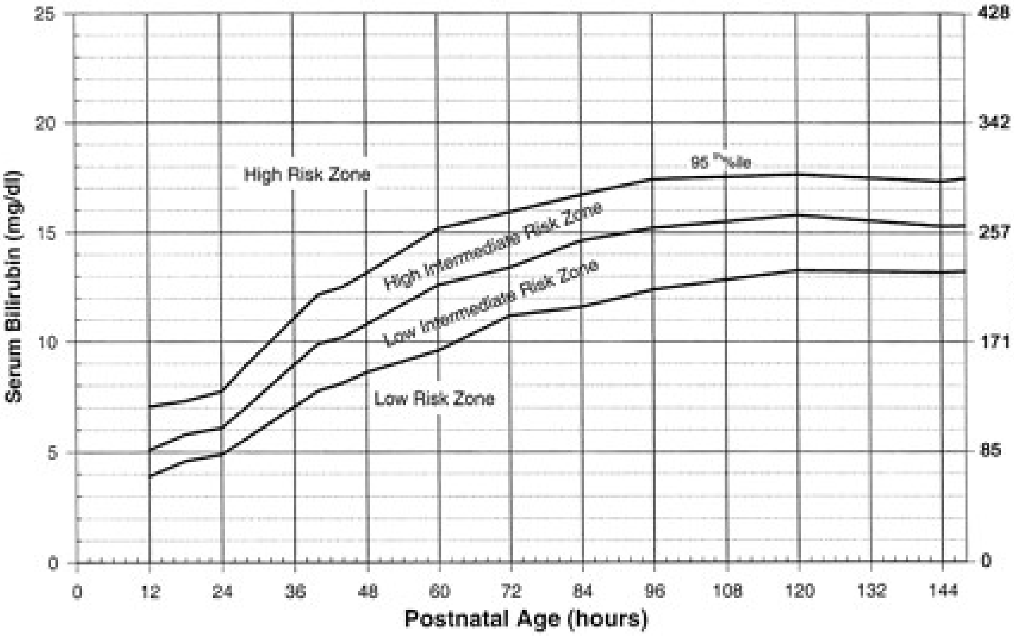

Figure: Nomogram for designation of risk in 2840 well newborns at 36 or more weeks' gestational age with birth weight of 2000 g or more or 35 or more weeks' gestational age and birth weight of 2500 g or more based on the hour-specific serum bilirubin values. Bhutani et al [37].

e) SAMPLE SIZE

Sample size was calculated based on incidence of pathological bilirubinaemia in ABO incompatibility in our neonatal set up. For correlation of 0.6, alpha error of 0.01and beta error of 0.01 the sample size was calculated to be 127. A total of 136 cases with incompatible blood group were studied.

1. INCLUSION CRITERIA

1) A+ve or B +ve babies born to O+ve mothers.

2) New born of GA $\geq$ 37 wks

3) New born of B.wt $\geq$ 2.5 kg and $\leq$ 4kg

4) Mothers undergoing LSCS for indications not causing hyperbilirubinemia in new born

5) Apgar >7 in newborn

a. EXCLUSION CRITERIA

2. Neonatal problems causing hyperbilirubinemia like birth asphyxia, sepsis, birth trauma, congenital malformation, etc.

3. Significant diseases in mother, which can cause hyperbilirubinemia in newborn, like GDM.

f) MANOEUVRE

The study was conducted in the Institute of Obstetrics and Gynaecology, Egmore. 212 babies born of caesarean section fulfilling the criteria were randomly recruited for the study. After initial stabilization of baby 3 mililitres of cord blood was collected in 3 separate prepared bottles and sent to the Institute Of Child Health immediately for blood grouping, direct coomb's test, serum bilirubin, hemoglobin, and reticulocyte count estimation.

Mean while a thorough history was taken with special emphasis on those condition leading on to pathological hyperbilirubinemia. In neonatal history any neonatal complications were asked including the history of passage of meconium within 24 hours. Baby's birth weight, a detailed general examination to rule out congenital anomalies, presence of concealed hemorrhage like hematomas or ecchymosis, breast feeding history were obtained. All babies who develop any other risk factor other than ABO incompatibility were excluded from the study.

Of the 212 babies screened, 136 babies were at risk of ABO incompatibility (A or B group babies delivered to O positive mothers). The rest of the babies were O group. All the babies who were A or B positive were followed up daily for clinical jaundice. Serum bilirubin was repeated on the fourth day. If the baby developed significant clinical jaundice before the 4^{th} day then serum bilirubin was estimated and appropriate treatment was started if required.

SERUM BILIRUBIN ESTIMATION

Bilirubin in our biochemistry department was estimated by Diazo method. Reagents used are absolute methanol, hydrochloric acid, diazo reagent and standard solution of bilirubin. Serum was diluted with water and methanol was added in an amount insufficient to precipitate proteins, yet sufficient to ensure that all the bilirubin reacts with the diazo reagent. Bilirubin reacts with diazotized sulphanilic acid to produce azo bilirubin which was quantified by spectrophotometry.

RETICULOCYTE COUNT

Reticulocyte count was estimated by supravital staining with brilliant cresyl blue. Blood was mixed with brilliant cresyl blue and peripheral smear was examined for polychromatic RBCs.

HEMOGLOBIN ESTIMATION

Hemoglobin estimation in this study was done with the help of auto analyzer.

DIRECT COOMB'S TEST

Blood was collected in EDTA bottle. One drop of 2.5% suspension of red cells was placed on a clean labeled test tube. The red cells were washed 3-4 times with saline. Add 1-2 drops of AHG reagent. Mix and centrifuge at 1000 rpm for 1 minute. The tube was gently shaken to dislodge the cell button and read the results using a concave mirror. If the results were negative, the test tube was incubated for further 5 minutes at room temperature, centrifuged and looked for results. 1 drop of 5% IgG sensitized red cells were added to the negative tests. Look for agglutination, if a negative test was obtained the test result was invalid and has to be repeated.

Agglutination of red cells was a positive direct coomb's test. Control tubes should be read before final interpretation. An immediate positive reaction indicates presence of IgG coating antibodies. Reaction due to IgG becomes weaker after incubation. A positive reaction after 5 minutes indicates coating by complement components.

RESULTS

A total of 136 babies with the risk of ABO incompatibility were included in the study. 73% (99) of the babies developed clinical jaundice and nearly 10% (13) of cases developed pathological jaundice.

The data obtained was analysed as follows:

1. features of study population (sex, birth weight, blood group distribution)

2. Correlation of cord blood bilirubin with peak bilirubin values.

3. Receiver operated characteristic curve for cord bilirubin and cut off value for predicting pathological hyperbilirubinemia.

4. Correlation of cord blood hemoglobin with peak bilirubin values.

5. Receiver operated characteristic curve for cord hemoglobin and cut off value for predicting pathological hyperbilirubinemia.

6. Correlation of cord blood reticulocyte count with peak bilirubin values.

7. Cut off value of cord blood reticulocyte count for predicting pathological hyperbilirubinemia.

8. Correlation between direct coombs' test and pathological hyperbilirubinemia.

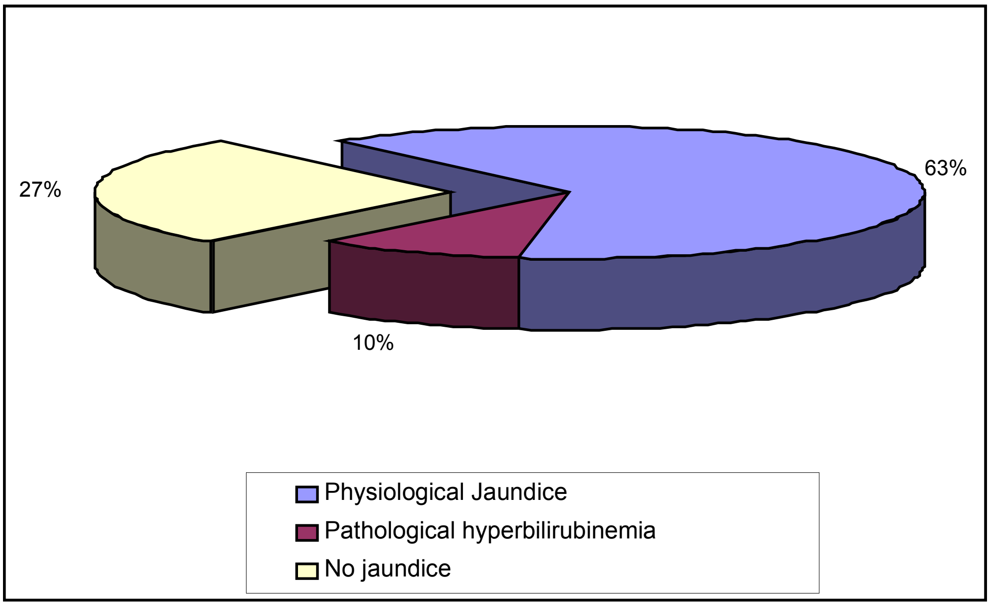

Figure 1: Incidence of jaundice and pathological hyperbilirubinemia

Out of the 136 babies who were at risk of ABO incompatibility, 73% (99) of the babies developed clinical jaundice and nearly 10% (13) of cases developed pathological jaundice.

SEX DISTRIBUTION

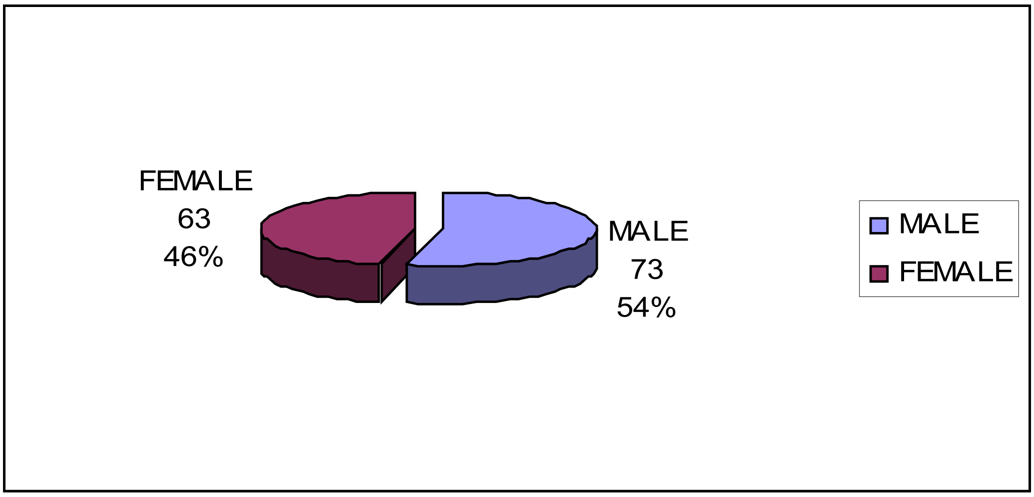

Figure 2: sex distribution in study population

Out of the 136 babies studied 73 were males (54%) and 63 were females (46%).

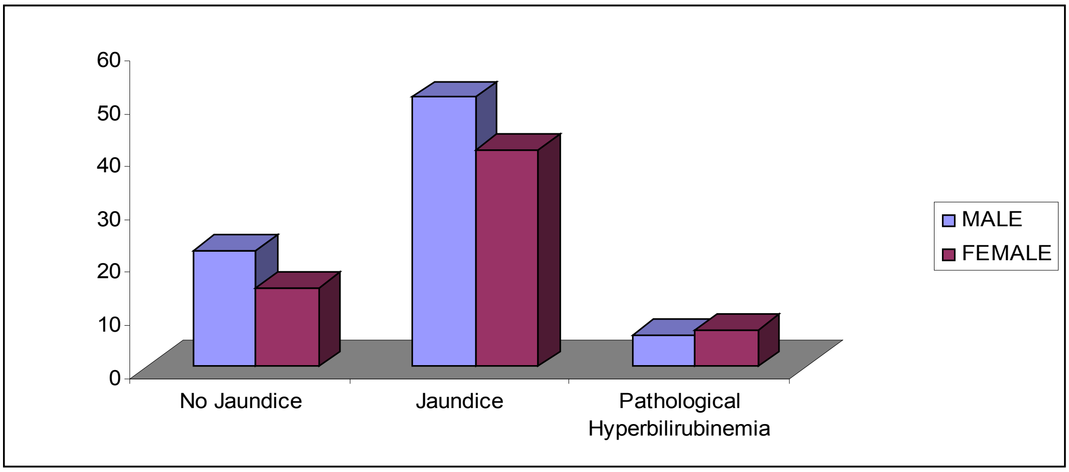

Figure 3: sex distribution in babies who developed clinical jaundice and pathological hyperbilirubinemia

The incidence of clinical jaundice and pathological hyperbilirubinemia was not significantly different in both sexes (P=0.92).

WEIGHT DISTRIBUTION

In our study group only babies between 2.5 to 4 kilograms were included (Appropriate for Gestational age)

Wt	No Jaundice	Jaundice	Pathological hyperbilirubinemia
2.5-3	26	60	8
3-3.5	10	35	4
3.5-4	1	4	1

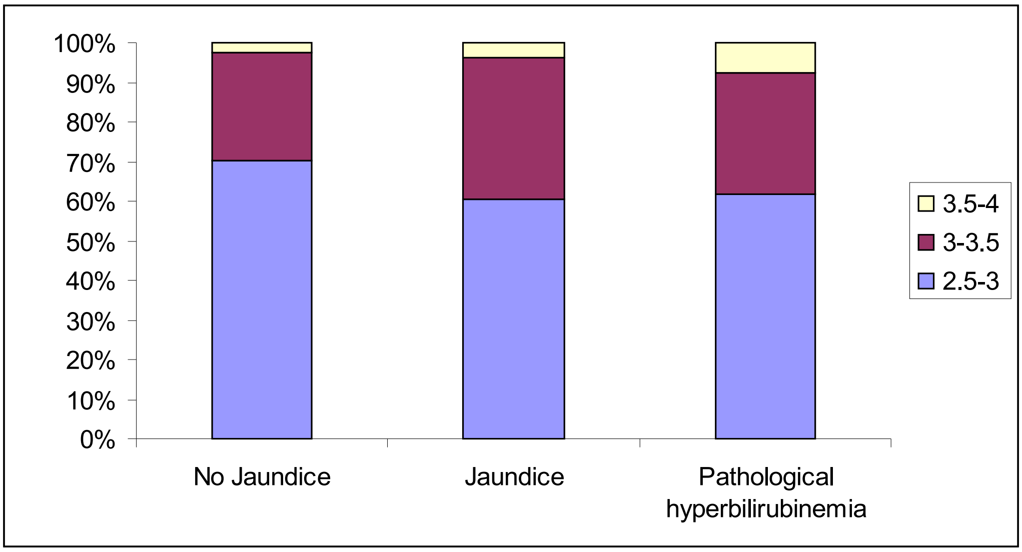

Figure 3: weight distribution

No significant difference in incidence of clinical jaundice or pathological hyperbilirubinemia was found between various weight groups (p value 0.54)

BLOOD GROUP AS A RISK FACTOR

Out of the 136 babies, 66 were A group (62 A positives and 4 A negatives) and 70 were B group (69 B positives and 1 B negative). Incidence of clinical jaundice B group (85%) was higher in compared to A group (74%). But this difference was not statistically significant (p value 0.91). No significant difference was there between the incidence of pathological hyperbilirubinemia also.

Blood group	No Jaundice	Jaundice	Pathological hyperbilirubinemia
A+	18	44	6
A-	2	2	0
B+	16	53	7
B-	1	0	0

CORRELATION BETWEEN CORD BILIRUBIN AND 4TH DAY BILIRUBIN

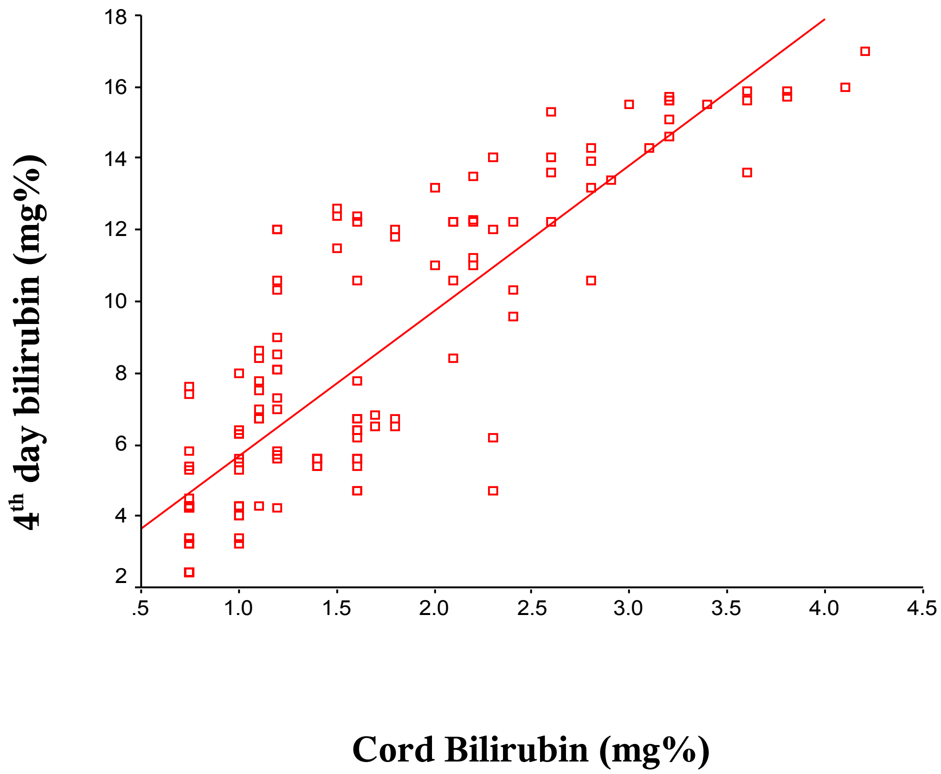

Figure 4: Correlation between cord bilirubin and 4th day bilirubin

Cord bilirubin has excellent correlation with the 4th day bilirubin levels. Pearson's correlation, $r = 0.86$ (p-value < 0.001).

For who were started on phototherapy before the 4th day, a corresponding 4th day value based on nomagram was used for statistical analysis.

PREDICTIVE VALUE OF CORD BILIRUBIN LEVELS

Cord bilirubin (mg%)	**Sensitivity**	**Specificity**
≥.8	100.00%	0.00%
≥1	100.00%	24.39%
≥ 1.1	100.00%	35.77%
≥1.2	100.00%	42.28%
≥1.4	100.00%	53.66%
≥1.5	100.00%	57.72%
≥1.6	100.00%	60.16%
≥1.7	100.00%	71.54%
≥1.8	100.00%	73.17%
≥2	100.00%	76.42%
≥2.1	100.00%	78.05%
≥2.2	100.00%	81.30%
≥2.3	100.00%	85.37%
≥2.4	100.00%	88.62%
≥2.6	100.00%	91.06%
≥2.8	92.31%	93.50%
≥2.9	92.31%	96.75%
≥**3**	**92.31%**	**97.56%**
≥3.1	84.62%	97.56%
≥3.2	84.62%	98.37%
≥3.4	53.85%	99.19%
≥3.6	46.15%	99.19%
≥3.8	30.77%	100.00%
≥4.1	15.38%	100.00%
≥4.2	7.69%	100.00%
≥4.2	0.00%	100.00%

RECIEVER OPERATED CHARACTERISTIC CURVE

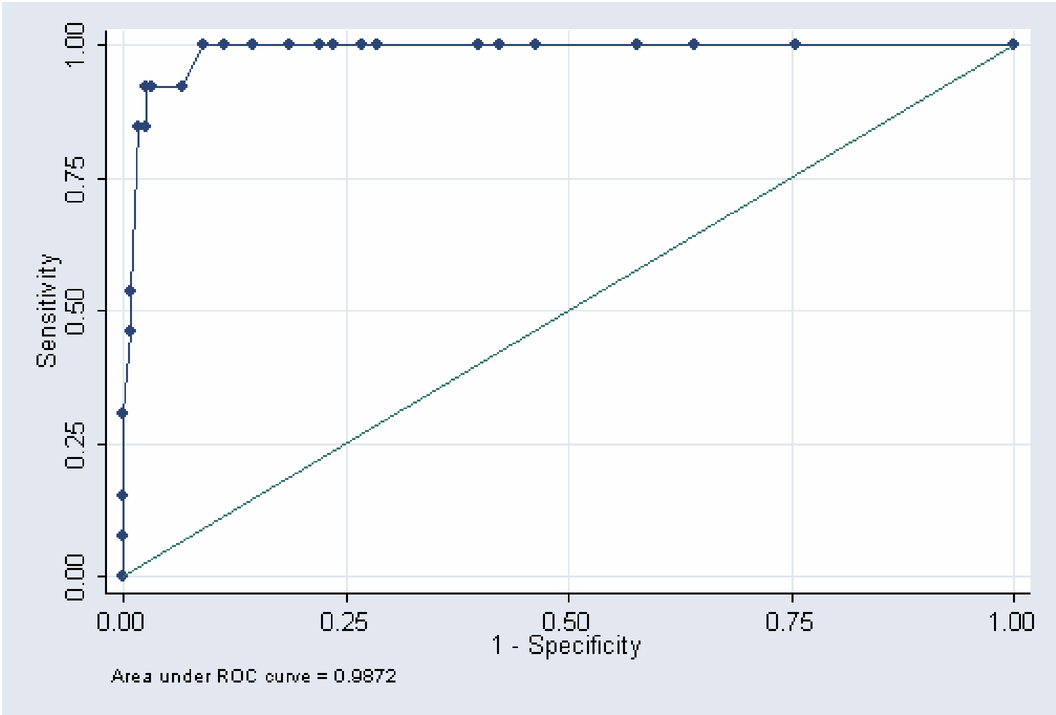

Figure 5: ROC curve for cord bilirubin

Area under the curve is 0.9872 which means that a randomly selected child with hyperbilirubinemia has a score larger than that for randomly chosen child without hyperbilirubinemia 98% of the time.

A value of ≥ 3 mg/dL can be used as a cut off for predicting pathological hyperbilirubinemia with a specificity of 92.3%, sensitivity of 97.5%, positive predictive value of 84.6 % and negative predictive value of 98.4 %

CORD BLOOD HEMOGLOBIN AND 4TH DAY BILIRUBIN

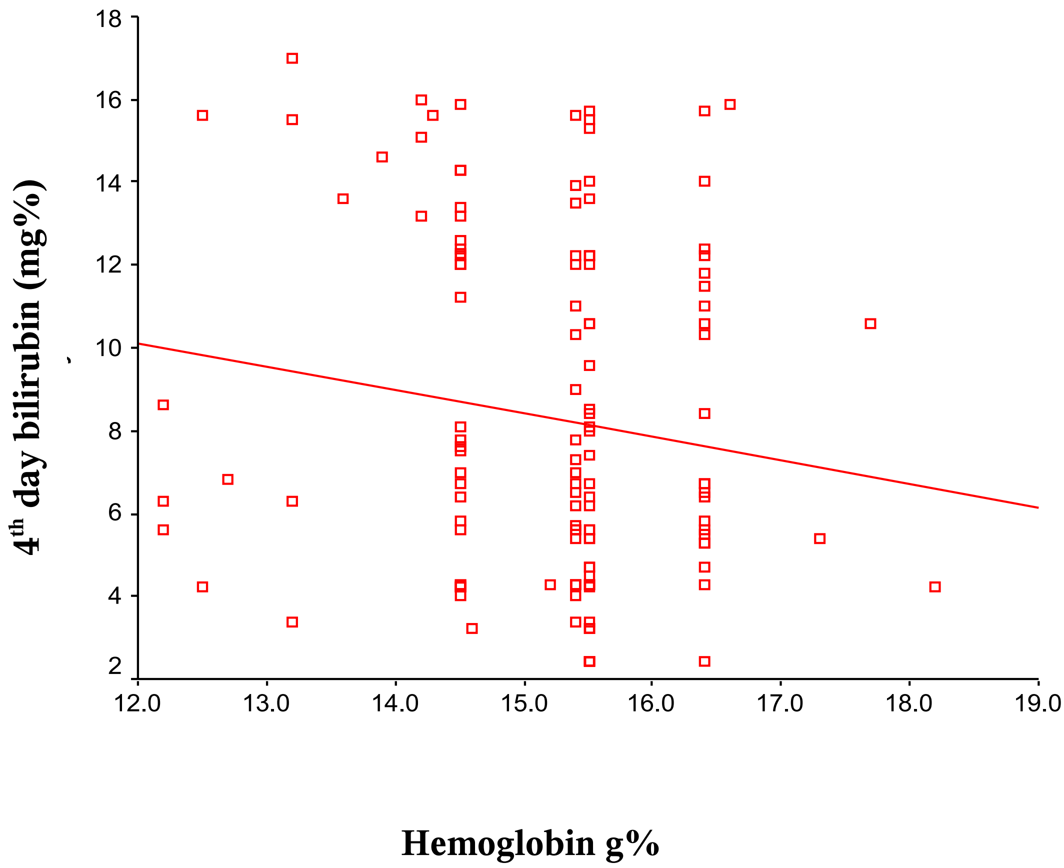

Figure 6: Correlation between cord hemoglobin and 4th day bilirubin

Lower cord hemoglobin is associated with higher risk of hyperbilirubinemia. The strength of the association is weak - Pearson's correlation r = - 0.139 (p-value=0.11).

PREDICTIVE VALUE OF CORD HEMOGLOBIN LEVELS

Cord hemoglobin (g %)	Sensitivity	Specificity
11.2	.000	.000
12.35	.000	.024
12.6	.077	.033
12.95	.077	.041
13.4	.231	.057
13.75	.231	.065
14.05	.231	.073
14.25	.385	.081
14.4	.462	.081
14.55	**.538**	**.293**
14.9	.538	.301
15.3	.538	.309
15.45	.615	.496
15.95	.846	.805
16.5	.923	.976
16.95	1	.976
17.5	1	.984
17.95	1	.992
19.2	1	1

RECIEVER OPERATED CHARACTERISTIC CURVE

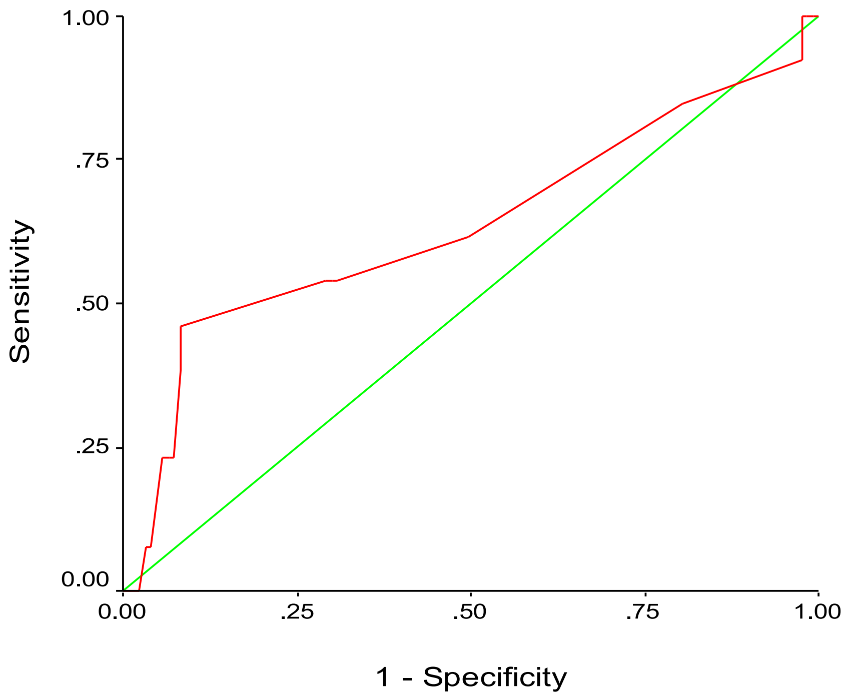

Figure 7: ROC curve for cord hemoglobin

Area under the curve was .633. A hemoglobin value below 14.55g/ dL can be used as a good predictor from this curve with a sensitivity of 53.8 %, specificity of 70.7 % positive predictive value of 15.9 % and negative predictive value of 93.4 %

CORRELATION BETWEEN RETICULOCYTE COUNT AND 4^TH^ DAY BILIRUBIN

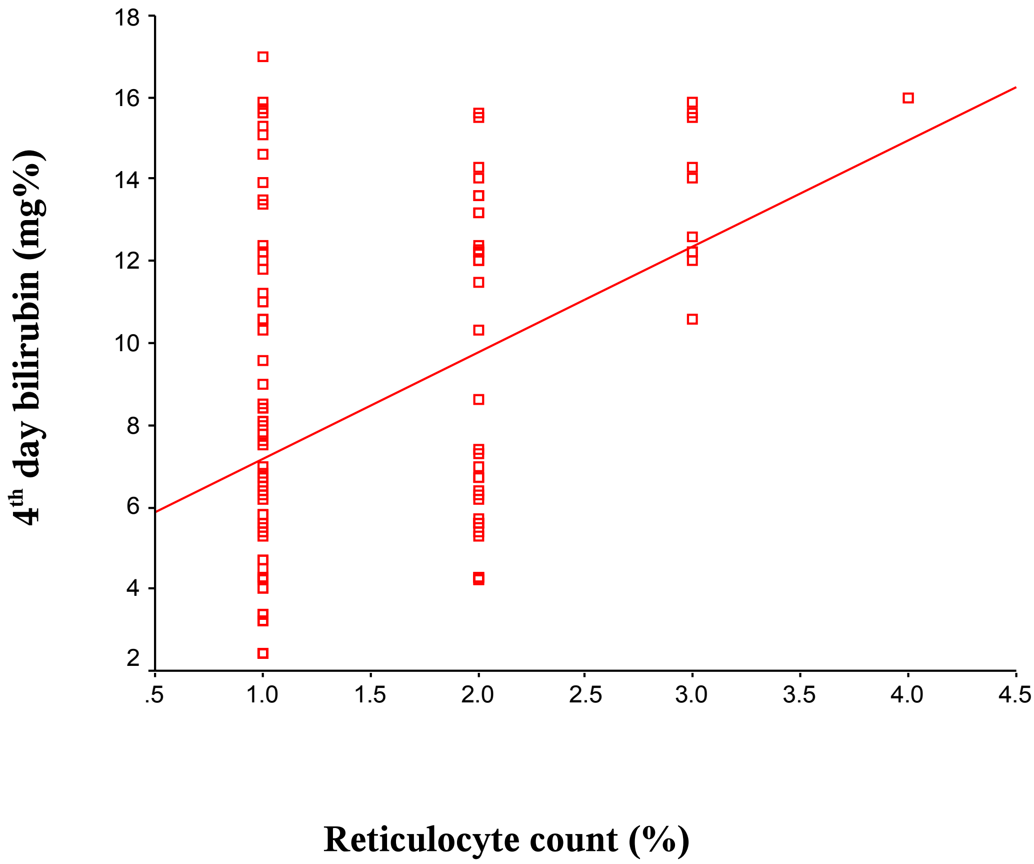

Figure 8: Correlation between cord reticulocyte count and 4th day bilirubin

The risk of hyperbilirubinemia increases with an increase in reticulocyte count p-value = 0.002. The strength of the correlation was weak Spearman's correlation r = 0.364 (p-value <0.01).

Cord reticulocyte count (%)	Sensitivity	Specificity
≥ 1	1.00	.000
≥ 2	**.462**	**.667**
≥ 3	.308	.943
≥ 4	.077	1
≥ 5	.000	1

A reticulocyte count ≥2% could predict the risk of pathological hyperbilirubinemia with a sensitivity of 46 % and specificity of 67%.

DIRECT COOMBS TEST AND PATHOLOGICAL HYPERBILIRUBINEMIA

Direct coomb's test	Pathological hyperbilirubinemia	
	Present	Absent
Positive	2	0
Negative	11	123

Direct coomb's test was positive in only 1.5 % of the babies studied. It was positive in 15.4 % of babies who developed pathological hyperbilirubinemia. All children who had positive Direct coombs test developed pathological hyperbilirubinemia. P-value = 0.008

DISCUSSION

The major cause of pathological hyperbilirubinemia is ABO incompatibility [1]. This study was conducted to find out whether routine cord blood analysis can be useful in predicting pathological hyperbilirubinemia in newborns at risk of pathological hyperbilirubinemia. The aim was to find out whether cord blood bilirubin, hemoglobin and reticulocyte count values correlated with the peak bilirubin values. If these values could predict the development of pathological hyperbilirubinemia we could decide on the early discharge of these at risk babies.

This study included 136 babies who were at risk of ABO incompatibility which include babies with either A or B blood group born to O positive mothers. All babies were term (>37 weeks) and appropriate for gestational age (2.5 – 4 kg). Those babies who had other potential causes for developing jaundice like birth asphyxia, sepsis, birth injuries, mother with diabetes, PIH were excluded from the study. Preterm babies were excluded from the study because the serum bilirubin levels and the peak level going for kernicterus were highly variable. The aim was to find out cord blood analysis is useful in predicting pathological hyperbilirubinemia.

Factors in the cord blood that we studied included cord bilirubin, hemoglobin, reticulocyte count and direct coomb’s test. Higher cord bilirubin levels, lower cord hemoglobin, higher reticulocyte count and a positive direct coomb's test were associated with a higher risk of the babies to develop pathological hyperbilirubinemia. In our study we tried to determine the correlation between cord blood bilirubin, hemoglobin, reticulocyte count and direct coomb's test positivity with the development of pathological hyperbilirubinemia. Final outcome measurement is pathological bilirubinaemia which is defined in our study as a 4^{th} day bilirubin value above 15 mg/dL or a serum bilirubin level more than the 95^{th} percentile for the age in hours.

13 out of 136 babies studied in our study developed pathological hyperbilirubinemia in our study (9.56 %). Bilirubin peaking was mostly noted in the 3^{rd} and 4^{th} day. Out of the 13 babies who had pathological hyperbilirubinemia 12 required phototherapy (phototherapy started on 3^{rd} or 4^{th} day) and 1 required exchange transfusion (on 3^{rd} day of life). The baby who required exchange transfusion had a cord blood bilirubin of 4.2mg/dL, cord blood hemoglobin 13.2mg/dL. But her reticulocyte count was 1 % and direct coomb's test was negative.

Cord bilirubin values in the study population were in the range of <1 to 4.2mg/dL, while that in the babies who developed hyperbilirubinemia was in the range of 2 – 4.2 mg/dL. The mean bilirubin level in babies with pathological hyperbilirubinemia was 3.1 mg/dL, where as those who didn't develop it was 1.31 mg/dL (p value <0.01).

Cord hemoglobin values in the study population were in the range of 12.2 to 18.2 mg/dL, while that in the babies who developed hyperbilirubinemia was in the range of 12.5 – 16.6 mg/dL. The mean hemoglobin level in babies with pathological hyperbilirubinemia was 14.73 mg/dL, where as those who didn't develop it was 14.62 mg/dL (p value 0.92). Hemoglobin was a poor predictor for the development of pathological hyperbilirubinemia.

Reticulocytosis in ABO hemolytic disease range from 6 % to 40 % in various studies [86, 85]. Reticulocytosis is seen in cord blood itself as the hemolysis in ABO incompatibility starts in utero itself. But in our study significant reticulocytosis was seen in none of the babies.

Direct coomb's test is usually negative or weakly positive in babies with babies with ABO incompatibility. In our study only two babies with pathological hyperbilirubinemia had Direct coombs test positivity (15.4 %). None of the babies without pathological hyperbilirubinemia had Direct coombs test positive.

CORRELATION OF VARIOUS CORD BLOOD ANALYSIS WITH HYPERBILIRUBINEMIA

Previous studies conducted showed a good correlation between cord blood bilirubin and the development of pathological hyperbilirubinemia. The cord bilirubin values which can predict pathological hyperbilirubinemia range from 1.7 mg/dL (Knupfer M, Pulzer F et al [79]) to 5 mg/dL (Whyte J, Graham H [77]) in various studies. The correlation of hemoglobin and reticulocyte count is not well studied. Cord hemoglobin level below 11 mg/dL to 11.5 mg/dL is associated with significant morbidity and mortality in babies with pathological hyperbilirubinemia[1].

In our study cord bilirubin has excellent correlation with the 4th day bilirubin levels Pearson's correlation, $r = 0.86$ (p-value < 0.001). So cord bilirubin can effectively predict the risk of pathological hyperbilirubinemia. This results are similar to that of studies done by Whyte J, Graham H [77]Chen JY, Ling UP et al [74]Procianoy RS et al. [76].

Lower cord hemoglobin is associated with higher risk of hyperbilirubinemia. The strength of the association is weak – Pearson's correlation $r= - 0.139$ (p-value=0.11). The risk of hyperbilirubinemia increases with an increase in reticulocyte count p-value = 0.002. The strength of the correlation was weak Spearman's correlation $r= 0.364$ (p-value <0.01). But in our study the reticulocytosis in babies with ABO incompatibility was not in the pathological range for the newborns. DIRECT COOMBS TEST was positive in only 1.5 % of the babies studied. It was positive in 15.4 % of babies who developed pathological hyperbilirubinemia. All children with positive DIRECT COOMBS TEST developed pathological hyperbilirubinemia. P-value = 0.008

PREDICTIVE VALUE OF CORD BLOOD TESTS

A cord bilirubin value of ≥ 3mg/dL can be used as a cut off for predicting pathological hyperbilirubinemia with a specificity of 92.3%, sensitivity of 97.5%, positive predictive value of 84.6 % and negative predictive value of 98.4%.

A hemoglobin value below 14.55 g/dL can be used as a good predictor from this curve with a sensitivity of 53.8 %, specificity of 70.7 %, positive predictive value of 15.9 % and negative predictive value of 93.4 %.

A reticulocyte count ≥2% could predict the risk of pathological hyperbilirubinemia with a sensitivity of 46 % and specificity of 67%. Direct coomb's test is positive in 15.4 % of babies who developed pathological hyperbilirubinemia. All children who had positive DIRECT COOMBS TEST developed pathological hyperbilirubinemia. (P-value = 0.008)

CONCLUSION

- ✓ Cord blood analysis is useful for predicting pathological hyperbilirubinemia in babies at risk of ABO incompatibility.
- ✓ In cord blood analysis cord bilirubin is the best predictor for the development of hyperbilirubinemia.
- ✓ Neonates with cord bilirubin values $\geq$ 3 mg/dL are at higher risk for developing pathological hyperbilirubinemia.
- ✓ A lower cord blood hemoglobin level is associated with a higher 4^{th} day bilirubin level.
- ✓ A cord hemoglobin level below 14.55 g/dL was associated with a higher risk of babies to develop pathological hyperbilirubinemia.
- ✓ Significant reticulocytosis in cord blood was not seen in babies with the risk of ABO incompatibility.
- ✓ Though Direct coomb's test was positive only 15 % of babies with pathological hyperbilirubinemia, all babies with positive direct coomb's test developed pathological hyperbilirubinemia.

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ANNEXURE

PROFORMA

Sl no:

Mothers name: Age: IP no. :

Baby's blood group:

Details of mother:

LMP:

EDD:

Blood group: O+ve

Obstetric index:

Consanguinity:

H/o jaundice during pregnancy:

H/o GDM:

H/o PIH:

Fever/lymphadenopathy in antenatal period:

Addictions / drug intake:

Previous obstetric history:

Family history of jaundice / liver disease:

Previous history of neonatal jaundice:

If present intervention done:

Mode of delivery: normal / LSCS / Forceps

If LSCS, indication:

Induction of labour:

H/o foul smelling liquor/ maternal fever:

Examination of baby:

Apgar:

Maturity:

Birth weight:

Sex:

CVS:

RS:

P/A:

CNS:

Hematomas / ecchymosis:

Meconium passed – within 24 hours.

Delayed beyond 24 hours.

Lab results:

Cord bilirubin - Total:

Direct:

Cord direct coomb's test:

Cord retic count:

Hemoglobin:

Bilirubin done on any subsequent day:

4th day bilirubin:

Clinical details of baby:

Significant disease in early neonatal period:

Time of onset of clinical jaundice:

Interventions done:

Phototherapy

Exchange transfusion